

Characterization and Functional Analysis of the Promoter of RAGE, the Receptor for Advanced Glycation End Products*

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The receptor for advanced glycation end products, RAGE, is a member of the immunoglobulin superfamily of cell surface molecules differentially expressed on a range of cell types. Ligation of RAGE perturbs homeostatic mechanisms and, potentially, provides a basis for cellular dysfunction in pathologic situations in which its ligands accumulate. To understand factors underlying RAGE expression, we cloned the 5'-flanking region of the RAGE gene and characterized putative regulatory motifs. Analysis of the putative promoter region revealed the presence of three potential NF- κ B-like and two SP1 binding sites. Transient transfection of vascular endothelial and smooth muscle cells using chimeric 5'-deletion constructs linked to luciferase reporter revealed that the region -1543/-587 contributed importantly to both basal and stimulated expression of the RAGE gene. This region of the RAGE gene contained three putative NF- κ B-like binding sites and was responsible for increased luciferase activity observed when endothelial or smooth muscle cells were stimulated with lipopolysaccharide. DNase I footprinting assays and electrophoretic mobility shift assay revealed that two of the three NF- κ B-like binding sites (1 and 2) were likely functional and responsive to stimuli. Upon simultaneous mutation of NF- κ B-like sites 1 and 2, both basal promoter expression and response to stimulation with LPS, as measured by relative luciferase activity, were significantly diminished. These results point to NF- κ B-dependent mechanisms regulating cellular expression of RAGE and suggest a means of linking RAGE to the inflammatory response.

RAGE¹ (for receptor for advanced glycation end products), a member of the immunoglobulin superfamily of cell surface molecules, was initially identified and characterized as a cellular interaction site for advanced glycation end products or AGEs, ligands formed in diverse circumstances as a consequence of irreversible glycation/oxidation of proteins or lipids (1–6). AGE

formation occurs during normal aging, and to an accelerated degree in hyperglycemic conditions such as diabetes mellitus. In renal failure, enhanced glycation of β_2 -microglobulin occurs as a result of its markedly delayed clearance, one consequence of which, the formation of AGE- β_2 -microglobulin, is likely linked to the inflammatory bone and joint destruction characteristic of dialysis-related amyloidosis (7–10). AGE formation is also favored in other settings associated with the accumulation of amyloid proteins, such as in components of intracellular neurofibrillary tangles (11–13), as well as in extracellular amyloid- β peptide accumulations (14) characteristic of Alzheimer's disease. We hypothesized that expression of RAGE in a range of cells, including endothelial cells, vascular smooth muscle cells, mononuclear phagocytes, and certain neurons, as identified by immunohistochemistry and *in situ* hybridization studies (15, 16), places this molecule in prime position to mediate the pathogenic effects of AGEs.

Analysis of RAGE in tissues has suggested that expression of the receptor and consequences of RAGE binding to its ligands are likely to be regulated at multiple levels. Immunohistochemical studies of diabetic, AGE-rich vasculature demonstrated enhanced RAGE immunoreactivity in both the endothelium and vascular smooth muscle from renal arteries obtained from patients with diabetes, compared with basal levels of staining for RAGE in the renal vasculature of age-matched control individuals (17). In patients with renal failure and dialysis-related amyloidosis, enhanced staining for RAGE was noted in the macrophages that pervaded inflamed amyloid deposits containing AGE- β_2 -microglobulin (10). In other tissue studies, we demonstrated that RAGE expression was enhanced in developing neurons of the central nervous system. As the generation of AGEs was unlikely in that environment, we hypothesized that RAGE might interact with other ligands in that setting. We found that RAGE served as a neuronal receptor for amphoterin, mediating the neurite-outgrowth promoting effects previously attributed to amphoterin, and that both RAGE and amphoterin could be co-localized in neurons of the developing rat central nervous system (18–20). In pathologic settings in the central nervous system, we have demonstrated that amyloid- β peptide interaction with RAGE results in enhanced neural toxicity and microglial activation/migration (21). Consistent with these findings, neuronal RAGE expression is increased in affected neurons in Alzheimer's brain (21). Recent observations from our laboratory have suggested enhanced immunostaining for RAGE in vasculature affected by autoimmune/inflammatory disorders, such as systemic lupus erythematosus² and murine atherosclerotic plaques (22).

These observations suggested that the expression of RAGE was subject to cell-specific regulation based on microenvironmental cues. To test this hypothesis, the promoter of RAGE

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF001095.

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¹ The abbreviations used are: RAGE, receptor for advanced glycation end products; AGE, advanced glycation end product; BAEC, bovine aortic endothelial cell; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; RSMC, rat vascular smooth muscle cell; PCR, polymerase chain reaction; bp, base pair(s); ANOVA, analysis of variance.

² A. M. Schmidt, unpublished observations.

was identified and its initial functional characterization undertaken. Given the diverse circumstances under which RAGE expression was apparently altered, we studied the effects of the prototypic stimulus, lipopolysaccharide (LPS), in both cultured endothelial cells (23) and vascular smooth muscle cells (24). Here we demonstrate that the promoter of RAGE is responsive to stimulation with LPS in both endothelial cells and vascular smooth muscle cells. Furthermore, the presence of NF- κ B-like binding sites appears essential in both endothelial cells and smooth muscle cells for mediating this response.

EXPERIMENTAL PROCEDURES

Isolation and Sequencing of the 5'-Flanking Region of the RAGE Genomic Clone—A bacteriophage library (2.0×10^6 plaque-forming units) constructed by cloning human lung genomic DNA into the *Bam*HI sites of λ DASH 11 (Stratagene, La Jolla, CA) was screened by hybridization with a synthetic oligonucleotide probe (Ps-1) corresponding to nucleotides +17 to +101 of the human RAGE cDNA sequence (6). The probe was 5'-end-labeled with [γ - 32 P]ATP using T4-polynucleotide kinase. Lifts were prepared and hybridized in QuikHyb hybridization solution according to the manufacturer's instructions (Stratagene). Positive plaques were selected and purified. Southern blot analysis revealed that one of the plaques contained a 3.5-kilobase *Bam*HI insert, which hybridized with Ps-1 as well as another oligonucleotide probe (Ps-2) corresponding to +19 to +43 of the human RAGE cDNA (6). This insert was isolated and digested with *Hind*III. The resulting fragments were subcloned into plasmid pBluescript II SK(+) for DNA sequencing. DNA sequencing was performed by the dideoxy chain-termination method (25) with Sequenase (U. S. Biochemical Corp.). Vector-specific oligonucleotide T3 and T7 primers were used to obtain initial sequence information. The DNA sequence obtained for the 5'-flanking region of the RAGE gene is shown in Fig. 1.

Primer Extension Analysis—Primer extension analysis was performed as described (26) to map the transcriptional initiation site. Briefly, two synthetic oligonucleotide primers, Pe-5 corresponding to -104 to -83 of the 5'-flanking region of the RAGE gene (Fig. 1) and Pe-6 corresponding to +18 to +41 of the human RAGE cDNA (6), were end-labeled with [γ - 32 P]ATP and annealed to 5 μ g of the human lung poly(A)⁺ RNA (CLONTECH, Palo Alto, CA) at 58 °C for 20 min. After cooling to room temperature for 10 min, Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) was added to extend primers at 42 °C for 30 min. Dideoxy sequencing of the RAGE genomic clone using the same primers as above (Pe-5 and Pe-6) was carried out as DNA sequence ladders. The extension products alongside the DNA sequence ladders were analyzed on a denatured polyacrylamide gel (8%) and exposed to autoradiographic film overnight.

Preparation of Nuclear Extracts from Cultured Cells—Bovine aortic endothelial cells (BAECs, Ref. 5) and rat vascular smooth cells (RSMCs, generously provided by Dr. Abraham Rothman), were maintained in Dulbecco's modified Eagle's medium or M199 (Life Technologies, Inc.), respectively, containing fetal bovine serum (10%, Life Technologies, Inc.) and penicillin (100 units/ml) and streptomycin (100 μ g/ml), in humidified incubators containing CO₂ (5%). Prior to studies of basal/stimulated function of the RAGE promoter, BAECs or RSMCs were washed with phosphate-buffered saline and placed in medium as above, except that serum was omitted for 8 h prior to exposure to the indicated stimuli. Both BAECs (utilized at passage 1-3 for experiments) and RSMCs were treated with LPS (10 ng/ml, Difco). After 8 h at 37 °C, cell monolayers were washed twice with cold phosphate-buffered saline and scraped into cold phosphate-buffered saline (1.0 ml) and nuclear extracts were prepared as described (27). Briefly, the cell suspension was centrifuged for 5 min at 3,500 rpm at 4 °C. The cell pellet was then resuspended in 0.4 ml of cold buffer A (0.010 M (pH 7.9) HEPES, 0.010 M KCl, 0.0001 M EDTA, 0.001 M dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 15 min, and then Nonidet P-40 (10%, 0.025 ml) was added. After vortexing for 15 s, cell lysates were centrifuged for 30 min at 13,000 rpm at 4 °C, the supernatant was removed, and the nuclear pellet was washed once with buffer A as above (1 ml), and resuspended in 0.1 ml of ice-cold buffer B (0.020 M (pH 7.9) HEPES, 0.4 M NaCl, 0.001 M EDTA, 0.001 M dithiothreitol, and 0.001 M phenylmethylsulfonyl fluoride PMSF) for 15 min at 4 °C with constant agitation. The nuclear lysate was then centrifuged at 13,000 rpm for 10 min at 4 °C, and the nuclear extract collected. The concentration of nuclear protein was determined at A₂₈₀ using an Ultra-Spec spectrophotometer (Pharmacia Biotech Inc.).

DNase I Footprint Assay—DNase I footprinting assay was employed

to determine the binding capabilities of the three putative NF- κ B-like binding sites in the 5'-flanking region of the human RAGE gene as revealed by computer analysis (Genetics Computer Group; Ref. 28). Three probes were amplified from the 5'-flanking region of the human RAGE gene by PCR with [γ - 32 P]ATP-labeled sense primer and unlabeled antisense primer. The first probe, containing NF- κ B-like binding site 1, spanned the region from -1581 to -738 of the 5'-flanking region. The second probe, containing NF- κ B-like binding site 2, spanned the region from -721 to -399 of the 5'-flanking region. The third probe, containing NF- κ B-like binding site 3, spanned the region from -587 to -399 of the 5'-flanking region. DNase I footprinting assay was performed according to previously published methods (29). For each DNase I nicking reaction, 30-50 ng of the 5'-end-labeled DNA fragments were diluted to ≈ 0.7 nM in a final volume of buffer (0.1 ml) containing 0.02 M Tris-HCl (pH 7.4), 0.002 M MgCl₂, 0.001 M CaCl₂, 0.0001 M EDTA, 0.040 M KCl, 100 μ g/ml bovine serum albumin, and 20 μ g/ml salmon sperm DNA, and preincubated with 2-10 gel shift units of purified human NF- κ B p50 (Promega) for 10 min on ice. DNase I (30 ng/ml) was added. After incubation for another 1 min at room temperature, reactions were terminated by the addition of stop reagent (0.05 ml) containing 0.1 M EDTA (pH 8.0), 0.8% SDS, 1.6 M ammonium acetate, and 300 μ g/ml salmon sperm DNA. After extraction with phenol:chloroform (1:1), nucleic acids were ethanol-precipitated, dried, and dissolved in 0.003 ml of formamide (96%, v/v) containing tracking dyes. Samples were then loaded onto a polyacrylamide gel (8%) containing urea (7 M) and electrophoresed. Dideoxy sequencing of the 5'-flanking region of the RAGE gene using the same sense primers as above was carried out as DNA sequence ladders.

Electrophoretic Mobility Shift Assay (EMSA)—Based on the results of DNase I footprinting assays indicating that only NF- κ B sites 1 and 2 were likely to be functional, two NF- κ B-like binding site oligonucleotide probes were created by annealing the oligonucleotides 5'-CTCGGAGG-GAGTTTCTGCTA-3' and 5'-TAGCAGAAACTCCCTCCGAG-3' for NF- κ B site 1 and 5'-AGAGTGGGGAACCCCTCCCA-3' and 5'-TGG-GAGGGGTTCCCCACTCT-3' for NF- κ B site 2 and end-labeled with [γ - 32 P]ATP. Approximately 150 pg of the labeled probes was incubated with 20 μ g of nuclear extracts at room temperature for 30 min. For competition assays, nuclear extracts were first incubated with 100-fold excess of the unlabeled oligonucleotides for 15 min at room temperature and then incubated with the end-labeled oligonucleotides for 30 min under the same condition as above. To test if sequences from the NF- κ B binding site 1 and 2 competed with consensus probe, nuclear extracts obtained from HeLa cells (Promega; Ref. 30) were preincubated with the indicated concentration of the RAGE promoter-specific unlabeled oligonucleotides for 15 min at room temperature, and then incubated with the end-labeled consensus NF- κ B probe for 30 min as above. For supershift assays, antibody to p50, p65 or c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA) was preincubated with nuclear extract from stimulated BAECs or RSMCs using the same conditions as described above, prior to the addition of the labeled binding site oligonucleotides. Samples were then loaded onto polyacrylamide gels and run with buffer containing 0.09 M Tris borate (pH 8.3) and 0.0025 M EDTA at 10 V/cm for 4 h.

5'-Deletion Plasmids of the RAGE Promoter—The constructs utilized for transfection experiments were created by cloning into the pGL3-Basic vector (Promega) different fragments of DNA from the 5'-flanking region of the human RAGE gene, either by using compatible restriction sites or by amplifying the specific regions with PCR. The PCR primers used in this study were designed according to the sequence of the 5'-flanking region of the RAGE gene (Fig. 1), and all inserts derived from PCR amplification and cloned into the pGL3-Basic vector were sequenced on both strands.

Transfection Experiments—Cells were isolated and grown/maintained as described above. Transfection of BAECs and RSMCs was performed with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's recommendations. Briefly, 2×10^5 cells were plated into the wells of six-well tissue culture plates (Corning, Corning, NY) 1 day prior to transfection. The DNA mixture was composed of test plasmid (2 μ g) and pSV β -galactosidase (0.5 μ g), which served as an internal control to normalize activities of luciferase. Cells were exposed to the mixture of LipofectAMINE and plasmid for 5 h. Following removal of the transfection reagent, fresh medium was added and the incubation continued for 48 h. Prior to studies of basal/stimulated function of the RAGE promoter, BAECs or RSMCs were washed with phosphate-buffered saline and placed in medium as above, except that serum was omitted for 8 h prior to exposure to stimuli. Where indicated, stimulus (LPS) was added to the medium 8 h prior to harvesting. Luciferase activity was determined using standard reagents (Promega)

FIG. 1. Nucleotide sequence of the first exon and 5'-flanking region of the human RAGE gene. +1 refers to the transcription start site determined by primer extension analysis as described under "Experimental Procedures." Three potential NF- κ B-like binding sites (starting at -1518, -671, and -467) and other potential transcription factor binding sites are underlined. The numbers above the single vertical lines represent the nucleotide at the 5'-end, and the numbers above double vertical lines represent the nucleotide at the 3'-end of the constructs used in this study. The bold arrow indicates the start of translation.

-1581 TCATCTTAAACCAATAGAATC
NF κ B #1

-1543

-1560 GCTCGGTGGACGAGAGT|GTCTGACTCAGATATCTACCTCGGAGGGAGTTT
-1510 CTGCTACTTTAGGGGAATTATTGACTGGGCTTTGGGGTTGAACCTTTTTTTT
-1460 TTTTAAAGAAAGAAAAAGAAACCCTGGGATCCATCTATTTTTTTTGTGT
-1410 TGTGTGTTTTTGTGTTGGTGGTGGTGGTGGTGGTGGTGGTCTTAATTT

-1360 TTAATTTAGTTTGGGGAAGTAGCTTGTTTTTTTTTTATAAATATGTTGA
-1310 TTTCTTGTCTTTTTTTTTTATTCTTACTTTCCCATATTAGGGGTAGCCA
-1260 TGAAAGGGGTACTGGTAAGAGAAAGGGGGACAAACAGAACTGGTAAAGAG
-1210 GCCCCCTGGCTCCAGGCCTGTCCATCAGGAAGTAAATTTTACAGGGCAC
-1160 CAAGCTTTGCCCCCTAAAATCCCTTAGGTGTTCTTTGTTTCATGCAGGCAG
-1110 GTTCTGCCGCAATTGATGTGGAGGCAGTGAAGGGCTTGGCCTGTCTGGCC
-1060 TCTCATCCCCCTTCTCCACAACCCTTGGGCAGGGCTGGACTCAGTAAT
-1010 TTTGAGGAAATTGAAGATGCCATCTTCCCTGTGAGTGACATGTCTTTAA
-960 TTTTTTAAAAAATACTATTTGAAAATTGGAGGGGGAAGAATGGGAAGGG
-910 AGTTATTGCCAAATATGTTAAATATGGGTTGGGGTGCTTGTATATGTATC
-860 TTCCTCAATTTCCCATAAATGAGGTATCTTTTTGTACACCAAAATCAA
-810 GGGGTAGGGAGAGGGAGGAGTTGCAAAAAGCCAGATGTGGGGGAAAAAGT

-738

-760 AACATCAACACTGTCCCATCTCT|CAGCCCTGAAGTACGTACCATCTGATCC
NF κ B #2

-710 CCTCAGACATTCTCAGGATTTTACAAGACTGTCAGAGTGGGGAAACCCCTC
-660 CCATTAAGATCCGGGCAGGACTGGGACAGGTGGAAGTGTGATGGGTGG

-587

-610 GGGGGTGGGAGGCATGGGCCGGG|GGCAGTTCTCTCTCACTTGTAACCTT
-560 GTGTAGTTTCACAGAAAAAACAATAATGCAGTTTAAATAAAGAAATTT

NF κ B #3

-510 CTTTTTCCCTGGGTTTAGTTGAGAATTTTTTCAAAAAACATGAGAAAC
TCF-1

-460 CCCAGAAAAAATGATTTTCTTTTACGAAGTTCCAAACAGGTTTCTCTC
AP-2 γ -IRE

-410 CTGTTCCCGACGCTTGCCTTCATGATGCAGGCCCAATTGCACCCTTGCA
TCF-1 NF-IL6

-360 ACAACAGTCTGGCCTGAACCTATTGATGCAACTTTGGCGCAATCAAGATG
AP-2 NF-IL6

-310 GGGCTCCAGTGGGTACCAGGCCCTGATGGACTGATGGAATAAATAG
NF-IL6 AP-2

-260 GATCGGGGGCTCTGAGGGAATGAGACCCTAGAGGGTACACTCCCCATCCC
AP-2 -202 γ -IRE SP1 AP-2

-210 CCAGGGAA|GTGACTGTACCCAGAGGCTGGTAGTACCAGGGGTGGGGTGA
TCF-1 γ -IRE

-160 TAATTATTCTCTAGTACCTGAAGGACTCTGTGCCAAAGGCATGAATTC
NRE-Box1

-110 CTAGCATTCCCTGTGACAAGACGACTGAAAGATGGGGCTGGAGAGAGGG
-55 SP1 TCF-1

-60 TGCA|GCCCCACCTAGGGCGGAGGCCACAGCAGGGAGAGGGGCAGACAGA
r-IRE +1

-10 GCCAGGACCTGGAAGGAAGCAGGATGGCAGCCGGAACAGCAGTTGGAGC
+49

+41 CTGGGTGCT|GGTCTCAGTCTTGGGGTGAGCCACTCCCTCAACCCCACTG
+91 ACCCTCCCTGCAGAAAGCACTTTAACCCCA

and measured in a luminometer (Wallac, Gaithersburg, MD) as directed, and results standardized for β -galactosidase activity.

Northern Blot Analysis—Acid phenol-guanidinium thiocyanate procedure (31) was used to isolate total RNA from BAECs or RSMCs in the presence or absence of stimulation with LPS for 8 or 16 h as indicated (after serum deprivation as above). The concentration of total RNA was determined by adsorption at $A_{260/280}$ and total RNA (20 μ g/lane) was subjected to electrophoresis. After electrophoretic separation on a formaldehyde gel (0.7%), the RNA was transferred to a Gene Screen hybridization filter (DuPont NEN) and linked by a UV cross-linker (Stratagene). The filter was then hybridized with the full-length human RAGE cDNA probe or glyceraldehyde-3-phosphate dehydrogenase cDNA probe labeled with [γ - 32 P]dCTP using the Random Primer Labeling System (Stratagene). After washing twice at room temperature (15 min/wash) with SSC (2 \times) containing SDS (0.1%) and once for 30 min at 60 $^{\circ}$ C with SSC (0.1 \times) containing SDS (0.1%), the filter was subjected to autoradiography overnight at -80 $^{\circ}$ C. Glyceraldehyde-3-phosphate dehydrogenase message was used as control to normalize to counts in the RAGE message by densitometry.

PCR-directed Mutagenesis—Mutation constructs were generated by PCR. The resulting PCR fragments were then subcloned into the pGL3-Basic vector and subjected to sequence analysis prior to transfection studies. pGL-B contains the wild-type construct, -1543 to +49. pGL-C, pGL-D, and pGL-G each contain the region -1543 to +49, but with the mutation(s) indicated below.

Statistical Analysis—To determine if differences between luciferase activity between the various constructs utilized in these studies were statistically significant, analysis of variance (ANOVA) was used.

RESULTS

Structural Analysis of the RAGE Promoter—Primer extension analysis was used to map the transcription start site. To identify multiple potential start sites, two primers were designed, Pe-5 corresponding to -104 to -83 of the 5'-flanking region of the human RAGE gene and Pe-6 corresponding to +18 to +41 of the human RAGE cDNA. An extended product of 41 nucleotides was obtained by using primer Pe-6 (Fig. 2), but no extended product was found by using primer Pe-5 (data not shown). These results indicated that the human RAGE gene has only one major transcription start site, a thymidine residue, designated as "+1." The translation start site is indicated by a bold arrow above the "A" of the ATG codon (Fig. 1).

Computer analysis (Genetics Computer Group; Ref. 28) was employed to match potential transcription factor binding sites in the RAGE promoter by comparison of the genomic DNA sequence upstream of the identified transcription start site with known transcription factor binding consensus sequences. The search revealed three putative NF- κ B-like consensus sequences (GDRRADYCCC) at -1518 to -1510 (referred to as 1), -671 to -663 (referred to as 2) and -467 to 458 (referred to as 3). Other general consensus sequences such as SP1, AP-2, γ -IRE, and NF-IL6 were also identified (Fig. 1). However, further examination of the sequence failed to identify classic

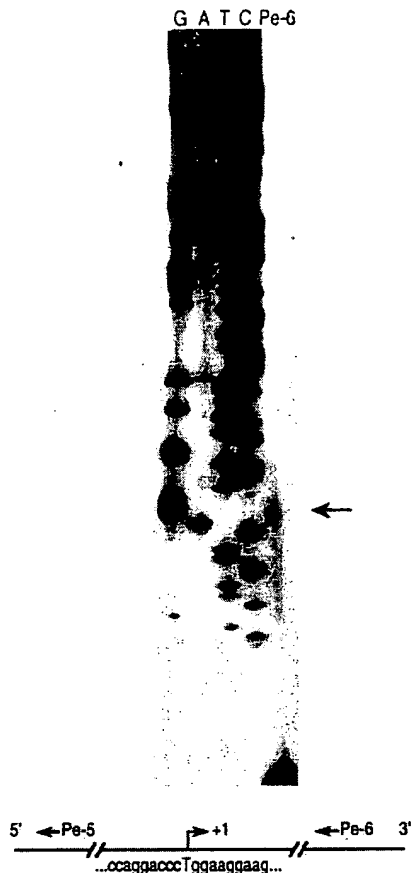


FIG. 2. Identification of the transcription start site using primer extension analysis. Two 5'-end-labeled oligonucleotide primers (Pe-5 and Pe-6) derived from either the 5'-flanking region of the RAGE gene or the human RAGE cDNA sequence were annealed to human lung poly(A)⁺ RNA (5 μ g). Primer-extended products were analyzed on a denatured polyacrylamide gel (8%) alongside a dideoxy sequencing reaction of the human RAGE genomic clone using the same primers as those which were used in the primer extension reactions. Shown in the figure is the schematic diagram of the primers used in both reactions and the mapping of the transcription start site. Primer-extended products were only obtained with Pe-6 as indicated in the figure, and not with Pe-5 (data not shown).

TATA and CAAT boxes at the appropriate positions.

To determine the functional capacity of the putative NF- κ B-like binding sites located within the RAGE promoter, DNase I footprinting analysis was performed. Using purified human NF- κ B p50, protection of only the first two NF- κ B-like binding sites (sites 1 and 2) was observed (Fig. 3). The first NF- κ B-like binding site spanned nucleotide coordinates -1518/-1510 (NF- κ B site 1), and the second spanned nucleotide coordinates -671/-663 (NF- κ B site 2) (Fig. 1). In contrast, addition of purified p50 failed to protect the third NF- κ B-like site, which spanned nucleotide coordinates -467/-458, even by increasing the amount of NF- κ B p50 (total amount of 10 gel shift units; data not shown). Consistent with these observations, the sequences of nucleotides comprising footprints at sites 1 and 2 were 95% and 50% identical to NF- κ B consensus sequences, respectively. Despite the fact that nucleotides comprising NF- κ B site 3 demonstrated 50% identity with NF- κ B consensus sequences, the lack of a footprint at this site indicates that other factors impact on its potential functional capacity, or that this site was not a functional site for transcription factor NF- κ B.

Functional Analysis of the RAGE Promoter: Basal Conditions—To assess which portions of the promoter were involved

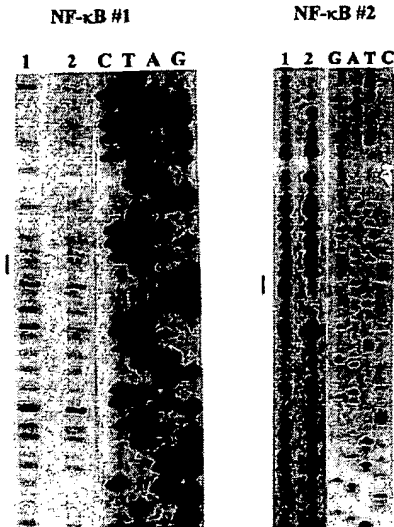


FIG. 3. Identification of NF- κ B binding sites using DNase I footprinting analyses. Three end-labeled DNA probes, generated by PCR with an end-labeled sense primer and an unlabeled antisense primer, were digested with DNase I after incubation with 2 gel shift units of human purified NF- κ B p50 (NF- κ B site 1, lane 2; NF- κ B site 2, lane 2) or without the purified transcription factor (NF- κ B site 1, lane 1; NF- κ B site 2, lane 1). Samples were analyzed on a denatured polyacrylamide gel (8%) alongside dideoxy sequencing reactions of the 5'-flanking region of the human RAGE gene with the same sense primers as those that were used to generate DNA probes. The vertical lines in *bold type* demonstrate areas protected by purified transcription factor NF- κ B p50 in NF- κ B binding site 1 and NF- κ B binding site 2. No protection by purified p50 was observed for putative NF- κ B site 3 (even after addition of 10 gel shift units of purified p50; data not shown).

in regulation of basal expression of RAGE, a series of chimeric 5'-deletion promoter-luciferase reporter constructs was developed (Fig. 4A). As indicated, pGL-B consisted of 1543 bp upstream of the transcription start site and pGL-E contained 738 bp upstream of the transcriptional start site. pGL-B-R, used as negative control, contained the same insert as pGL-B but in the opposite orientation. These 5'-flanking sequences of the human RAGE gene were cloned *upstream* of the luciferase gene in the pGL3-Basic vector (Fig. 4A). The resulting plasmids were then transfected into BAECs or RSMCs. 48 h after transfection, cell lysates were prepared and tested in the luciferase assay. pGL-3 control vector (Promega), a luciferase expression vector containing a SV40 promoter and enhancer, served as positive control. Efficiency of transfection was assessed by monitoring simultaneous transfection with another plasmid containing the β -galactosidase gene. (Thus, data are reported as normalized luciferase activity based on measurement of β -galactosidase.) Although the construct pGL-B demonstrated weaker basal promoter function than the construct pGL-E in both BAECs and RSMCs ($p < 0.05$ in both cases), transfection with pGL-B or pGL-E resulted in significant increases of luciferase activity in both cell types compared with the construct pGL-B-R ($p < 0.001$). These results indicated that both -1543/+49 and -738/+49 constructs contained motifs that contributed to basal expression in BAECs and RSMCs, although the latter construct seemed to have a greater promoter activity than the former under the present conditions.

To identify regions essential for basal promoter activity, we prepared three additional chimeric 5'-deletion promoter-reporter gene constructs, pGL-21 (containing 587 bp upstream of the transcriptional start site), pGL-22 (containing 202 bp upstream of the transcriptional start site), and pGL-23 (containing 55 bp upstream of the transcriptional start site) (Fig. 4A). These constructs were then transfected into BAECs and

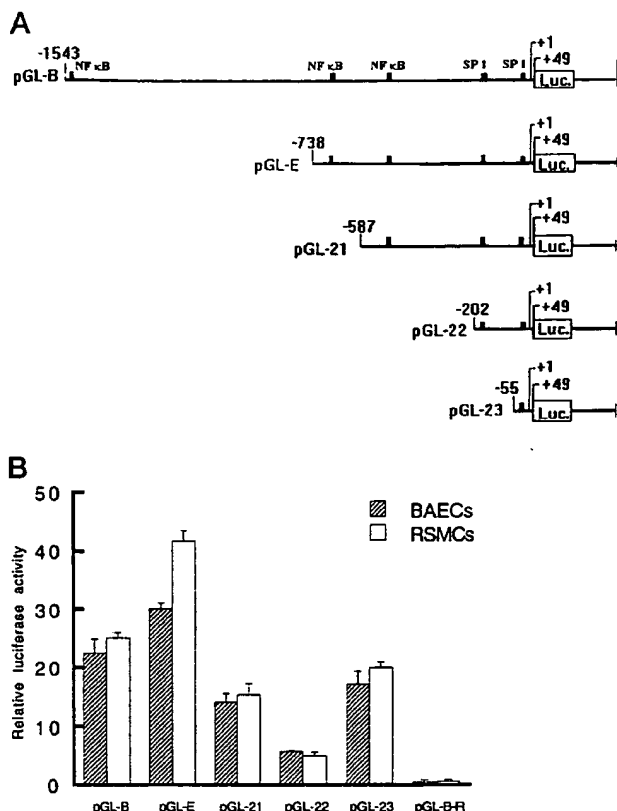


FIG. 4. Development of RAGE promoter luciferase chimeras: promoter function under basal conditions in BAECs and RSMCs. A, properties of the 5'-deleted RAGE promoter-luciferase reporter gene constructs. The relative size of the 5'-deletions are graphically represented and their end points shown. The transcription start site is designed as +1. Two putative NF- κ B-like binding sites identified in this study and two SP1 binding sites are represented by symbols located above the pGL-B reporter construct in the first line. B, basal promoter function for each 5'-deletion plasmid was studied by measurement of relative activities obtained by comparison of the luciferase activity of cells transfected with pSV- β -galactosidase control vector in BAECs and RSMCs. Data represent the mean \pm standard error obtained from three individual experiments. Statistical analysis (ANOVA) is as follows. BAECs: pGL-B versus pGL-E, $p < 0.05$; pGL-B versus pGL-B-R, $p < 0.001$; pGL-B versus pGL-21, $p < 0.01$; pGL-B versus pGL-22, $p < 0.01$; pGL-B versus pGL-23, $p < 0.05$; pGL-E versus pGL-21, $p < 0.01$; pGL-E versus pGL-22, $p < 0.01$; pGL-E versus pGL-23, $p < 0.01$; pGL-21 versus pGL-22, $p < 0.01$; pGL-21 versus pGL-23, $p > 0.05$; pGL-22 versus pGL-23, $p < 0.01$. RSMCs: pGL-B versus pGL-E, $p < 0.05$; pGL-B versus pGL-B-R, $p < 0.001$; pGL-B versus pGL-21, $p < 0.01$; pGL-B versus pGL-22, $p < 0.01$; pGL-B versus pGL-23, $p < 0.05$; pGL-E versus pGL-21, $p < 0.01$; pGL-E versus pGL-22, $p < 0.01$; pGL-E versus pGL-23, $p < 0.01$; pGL-21 versus pGL-22, $p < 0.01$; pGL-21 versus pGL-23, $p > 0.05$; pGL-22 versus pGL-23, $p < 0.01$.

RSMCs. Transfection of either pGL-21 or pGL-23 resulted in luciferase expression, although relative luciferase activity was significantly less than that observed with pGL-B or pGL-E. Furthermore, pGL-22 demonstrated strikingly diminished basal promoter activity compared with pGL-B and pGL-E in both cell types (Fig. 4B). These data indicated that the region 55 bp upstream of the start site contained motifs demonstrating promoter activity for RAGE, and that enhancement motifs for basal expression of the RAGE gene may exist within the -1543/-587 region. In contrast, negative regulatory elements are likely contained within the -202/-55 region.

Functional Analysis of the RAGE Promoter in the Presence of Stimulation with LPS—5'-Deletion constructs as above were used to transfect BAECs (Fig. 5A) or RSMCs (Fig. 5B) alone or in the presence of LPS. In both cell types, LPS stimulation

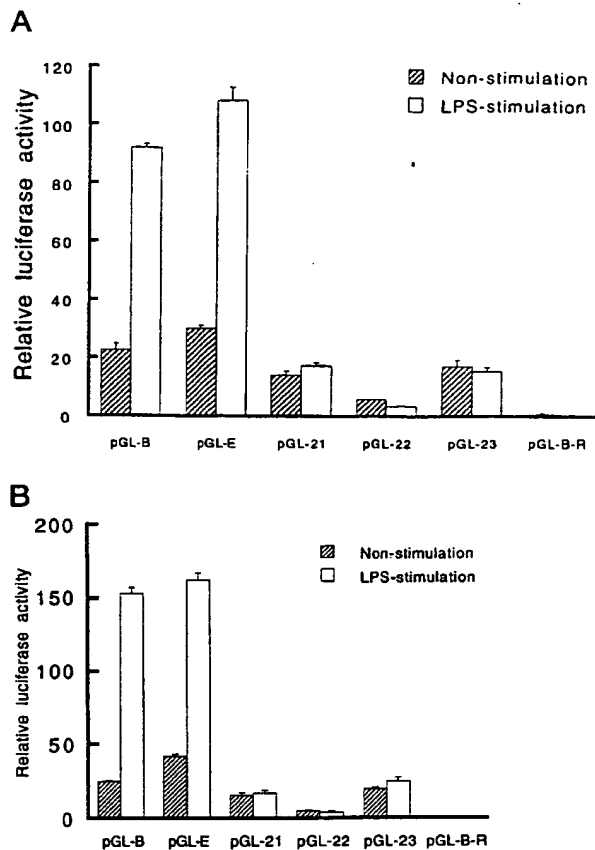


FIG. 5. Inducibility of promoter function and transcriptional activity: analysis by relative luciferase activity in BAECs (A) and RSMCs (B). A, relative luciferase activities were obtained in BAECs transiently transfected with 5'-deletion promoter-luciferase constructs under basal conditions or in the presence of LPS for 8 h as described under "Experimental Procedures." Statistical analysis: pGL-B \pm LPS, $p < 0.01$; pGL-E \pm LPS, $p < 0.01$; pGL-B + LPS versus pGL-E + LPS, $p > 0.05$; pGL-B + LPS versus pGL-21 + LPS, $p < 0.01$; pGL-B + LPS versus pGL-22 + LPS, $p < 0.01$; pGL-B + LPS versus pGL-23 + LPS, $p < 0.01$; pGL-B + LPS versus pGL-B-R + LPS, $p < 0.01$; pGL-21 \pm LPS, $p > 0.05$; pGL-22 \pm LPS, $p > 0.05$; pGL-23 \pm LPS, $p > 0.05$. B, relative luciferase activities were obtained in RSMCs transiently transfected with 5'-deletion promoter luciferase constructs either under basal conditions or in the presence of LPS under the same condition as above. Statistical analysis: pGL-B \pm LPS, $p < 0.01$; pGL-E \pm LPS, $p < 0.01$; pGL-B + LPS versus pGL-E + LPS, $p > 0.05$; pGL-B + LPS versus pGL-21 + LPS, $p < 0.01$; pGL-B + LPS versus pGL-22 + LPS, $p < 0.01$; pGL-B + LPS versus pGL-23 + LPS, $p < 0.01$; pGL-B + LPS versus pGL-B-R + LPS, $p < 0.01$; pGL-21 \pm LPS, $p > 0.05$; pGL-22 \pm LPS, $p > 0.05$; pGL-23 \pm LPS, $p > 0.05$.

significantly increased luciferase expression of the constructs pGL-B (~4.1-fold for BAECs and ~6.0-fold for RSMCs compared with basal conditions; $p < 0.01$ in both cases) and pGL-E (~3.7-fold for BAECs and ~3.8-fold for RSMCs compared with basal conditions; $p < 0.01$ in both cases). Unlike basal expression, no significant difference between the luciferase activity of pGL-B and pGL-E was identified in the presence of LPS stimulation ($p > 0.05$). Stimulation with LPS did not enhance expression of luciferase from constructs pGL-21, pGL-22, and pGL-23 ($p > 0.05$ in each case and in both cell types). Taken together, these data suggested that both pGL-B and pGL-E constructs contained positive regulatory elements, which were, at least in part, responsive to LPS-stimulation. Consistent with previous work, this finding was indeed significant, if not expected, given that this region likely contains two functional NF- κ B-like binding sites. However, given that other potential regulatory elements are also present in this region, it is possible that stimuli such as LPS may utilize multiple elements

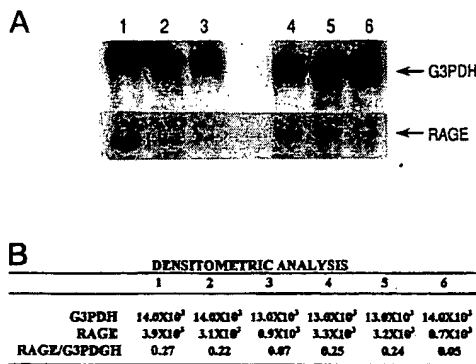


FIG. 6. Analysis of transcriptional activity of RAGE gene in LPS-stimulated BAECs and RSMCs by Northern blot. A, analysis of transcriptional activity of the RAGE gene in RSMCs (lanes 1–3) or BAECs (lanes 4–6) was performed by Northern analysis. In lanes 1 and 4, cells were incubated with LPS (10 ng/ml) for 8 h and in lanes 2 and 5, cells were incubated with LPS (10 ng/ml) for 16 h. In lanes 3 and 6, cells were incubated with bovine serum albumin (16 h; 10 ng/ml) as control. At the end of the incubation times, cells were harvested and RNA prepared as described above. The human RAGE cDNA probe or human glyceraldehyde-3-phosphate dehydrogenase cDNA probe was utilized (the latter as a quantitative control to normalize RAGE message results) and Northern blot analysis performed as described above. Each lane contains total RNA (20 μ g). Densitometric analysis was then performed and results reported in B.

within this promoter. In contrast, pGL-21, pGL-22, and pGL-23 contained elements that were more important in basal expression of the promoter (either positive or negative influence), rather than in stimulatory settings such as treatment with LPS.

To demonstrate that stimuli such as LPS may increase the level of RAGE transcripts in both RSMCs (Fig. 6, lanes 1–3) and BAECs (Fig. 6, lanes 4–6), Northern blot analysis was performed with total RNA from the LPS-treated BAECs or RSMCs. LPS stimulation for either 8 h (lanes 1 and 4) or 16 h (lanes 2 and 5) resulted in appearance of RAGE message in both cell types (Fig. 6). Compared with treatment of RSMCs with bovine serum albumin (lane 3), treatment of RSMCs with LPS resulted in \approx 3.8-fold and 3.1-fold increases in RAGE transcripts at 8 and 16 h after LPS, respectively. Similarly, compared with control albumin (lane 6), treatment of BAECs with LPS resulted in an \approx 5-fold and \approx 4.8-fold increase in RAGE transcripts at 8 and 16 h after LPS, respectively. The increase in levels of RAGE mRNA in stimulated cultures was consistent with the increase in transcription observed in experiments with chimeric promoter-reporter gene constructs (Fig. 5).

As demonstrated above, DNase I footprinting assays suggested that NF- κ B complexes were associated with DNA sequences in the region of the promoter containing putative NF- κ B-like binding sites 1 and 2. To further characterize the interaction of those putative binding sites with the NF- κ B complex and to delineate their role in the transcriptional regulation of RAGE, we performed EMSA. The synthesized oligonucleotides corresponding to the putative NF- κ B-binding sites 1 and 2 were end-labeled with [γ -³²P]ATP and incubated with nuclear extracts (20 μ g) from either unstimulated or stimulated RSMCs and BAECs. In BAECs, compared with the basal state (Fig. 7A, lanes 3, 4, 7, and 8) treatment with LPS (Fig. 7A, lanes 1, 2, 5, and 6) resulted in increased signal by densitometric analysis of \approx 2.1-fold for NF- κ B site 1, and \approx 2.7-fold for NF- κ B site 2, indicative of nuclear binding activity. In RSMCs, compared with the basal state (Fig. 7B, lanes 3, 4, 7, and 8), treatment with LPS (Fig. 7B, lanes 1, 2, 5, and 6) resulted in increases of \approx 4.5-fold for NF- κ B site 1, and \approx 4.7-fold for NF- κ B site 2. In each case, the specificity of the gel shift band for

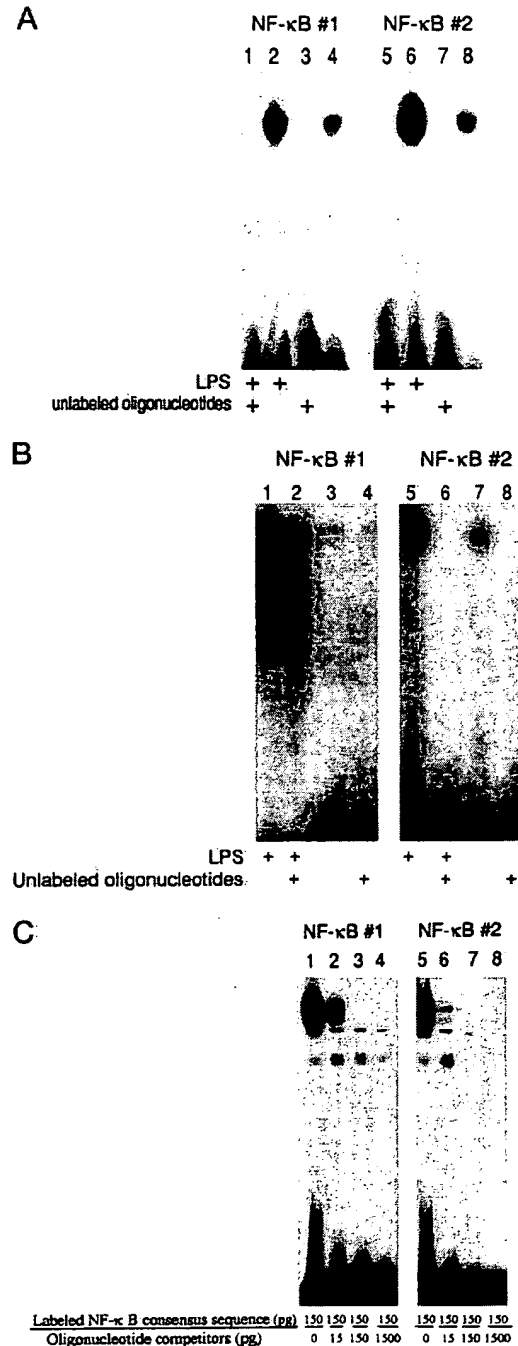


FIG. 7. Electrophoretic mobility shift assays of the two NF- κ B-like binding sites located within the promoter of RAGE. A and B, nuclear extracts (20 μ g) were prepared from BAECs (A) or RSMCs (B) treated with LPS for 8 h, and EMSA performed as described above. Lanes 1, 2, 5, and 6 represent nuclear extracts from cells that had been treated with LPS (10 ng/ml). Nuclear extracts from untreated cells are shown in lanes 3, 4, 7, and 8. In competition experiments, a 100-fold molar excess of unlabeled NF- κ B-like binding site oligonucleotides was preincubated with the nuclear extract as indicated in lanes 1, 3, 5, and 7 in A and lanes 2, 4, 6, and 8 in B. The protein/DNA complexes were then analyzed on 0.25 \times Tris borate EDTA native gels. C, HeLa cell nuclear extract was first preincubated with the indicated concentrations (lane 1, 0 pg; lane 2, 15 pg; lane 3, 150 pg; lane 4, 1,500 pg) of the RAGE promoter-specific NF- κ B-like binding site oligonucleotides, and then with end-labeled NF- κ B probe (150 pg).

NF- κ B was demonstrated by competition assays in which the nuclear extracts were preincubated with a 100-fold excess of the unlabeled NF- κ B-like binding site oligonucleotides (Fig. 7,

A, lanes 1, 3, 5, and 7; and B, lanes 2, 4, 6, and 8).

To demonstrate that both site 1 and site 2 NF- κ B-like binding sites competed with consensus probe for NF- κ B, electrophoretic mobility shift assays were performed using nuclear extracts from HeLa cells and a 32 P-labeled NF- κ B consensus probe in the presence of increasing concentrations of RAGE promoter-specific NF- κ B-like binding site oligonucleotides as unlabeled competitors. These data revealed that 15 pg of site 2 oligonucleotide almost completely removed the gel shift signal (>95%), while the same amount of site 1 oligonucleotide resulted in a 72% decrease of the original band (Fig. 7C). When 150 pg of the site 1 oligonucleotide was utilized, 95% of the signal was removed (Fig. 7C). These data suggested that both RAGE promoter-specific NF- κ B-like binding sites oligonucleotides competed with consensus probe, and that site 2 likely demonstrated somewhat higher relative affinity than site 1.

To investigate which members of the NF- κ B family were responsible for these findings (32–35), supershift assays were performed for NF- κ B sites 1 and 2. When the nuclear extracts from BAECs treated with LPS (10 ng/ml) were incubated with either anti-p65 IgG or anti-p50 IgG, a new more slowly migrating band appeared, with diminished intensity of the original band. In the presence of both anti-p65 and anti-p50 IgG, the original NF- κ B gel shift band (indicated by arrow) was virtually abolished and replaced by two more slowly migrating bands, suggesting that the DNA binding complex was composed of p50 and p65 members of the NF- κ B family (Fig. 8A). In RSMCs, when the nuclear extracts from cells treated with LPS (10 ng/ml) were incubated with either anti-p65 IgG or anti-p50 IgG, a new more slowly migrating band appeared, with reduced intensity of the original band. In the presence of both anti-p65 and anti-p50 IgG, the original NF- κ B gel shift band (arrow) was significantly reduced (Fig. 8B), indicating that the DNA binding complex was composed largely of p50 and p65. In contrast, incubation of the nuclear extracts from BAECs or RSMCs treated with LPS with anti-c-Rel IgG had no effect (data not shown).

To further study the role of the NF- κ B binding sites in transcriptional regulation of RAGE, PCR-directed mutagenesis was performed, altering NF- κ B sites 1 and 2 at residues known to be critical in mediating their interaction with the NF- κ B complex (Fig. 9A). Both the wild-type and mutant RAGE promoter-luciferase constructs, as delineated in Fig. 9A, were used to transfect BAECs and RSMCs. Under basal conditions, only when both NF- κ B-like binding site 1 and 2 were simultaneously mutated (pGL-G) did luciferase activity decrease (38% compared with pGL-B in BAECs ($p < 0.01$) and 43% compared with pGL-B in RSMCs ($p < 0.01$); Fig. 9, B and C, respectively). Mutation of either NF- κ B binding 1 or 2 (pGL-C and pGL-D, respectively) did not significantly alter basal luciferase activity. These data suggested that either NF- κ B binding site 1 or 2 could efficiently maintain the functional activity of the promoter under basal conditions.

Under stimulatory conditions (LPS), when NF- κ B site 1 was mutationally inactivated (pGL-C), an $\approx 14\%$ decrease in luciferase activity compared with pGL-B was observed in BAECs (Fig. 9B) and an $\approx 27\%$ decrease in luciferase activity compared with pGL-B was observed in RSMCs (Fig. 9C) ($p < 0.05$ in both cases). When NF- κ B site 2 was mutationally inactivated (pGL-D), an $\approx 28\%$ decrease in luciferase activity compared with pGL-B was observed in BAECs (Fig. 9B) and an $\approx 20\%$ decrease in luciferase activity compared with pGL-B was observed in RSMCs (Fig. 9C) ($p < 0.05$ in both cases). In contrast, when sites 1 and 2 were simultaneously mutated (pGL-G), a nearly complete loss of inducibility in the presence of LPS stimulation was seen ($\approx 78\%$ decrease in luciferase activity compared with

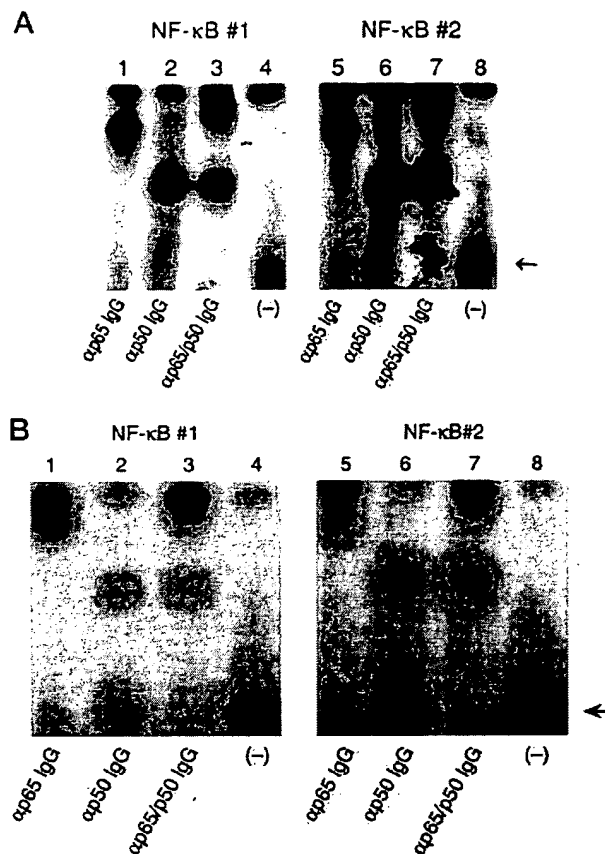


FIG. 8. EMSA/supershift assays to identify the members of NF- κ B family involved in binding to RAGE promoter. A, BAECs; B, RSMCs. In both BAECs and RSMCs, NF- κ B sites were studied alone (lanes 4 and 8) or in the presence of anti-p50 IgG (lanes 2 and 6), anti-p65 IgG (lanes 1 and 5), or both anti-p50/anti-p65 IgG together (lanes 3 and 7). In each case, final concentration of antibody employed was 10 μ g/ml and antibody(ies) were preincubated with nuclear extract from stimulated BAECs (A) or RSMCs (B). In A and B, arrow indicates the location of original gel shift band prior to supershift studies (lanes 4 and 8).

pGL-B was observed in BAECs and $\approx 83\%$ decrease in luciferase activity compared with pGL-B was observed in RSMCs; $p < 0.01$ in both cases, Fig. 9, B and C, respectively). Taken together, these data suggested that NF- κ B binding sites 1 and 2 alone, but especially in concert, are central regulatory elements in the RAGE promoter under stimulatory settings such as treatment with LPS.

DISCUSSION

We have reported the cloning and sequencing of the 5'-flanking region of the RAGE gene and identified a single major transcriptional start site. Basal promoter function was demonstrated by comparison of relative luciferase activities of specific constructs in bovine aortic endothelial cells and rat vascular smooth muscle cells. Studies designed to assess inducibility of the promoter using a prototypic stimulus revealed that the RAGE promoter responds to LPS in both of these cell types.

Our previous studies have indicated that two clinically important settings in which RAGE expression and induction of cellular oxidant stress appear closely linked are diabetic tissue, in which AGEs accumulate, and Alzheimer's brain, with deposits of amyloid- β peptide (17, 21, 36–39). In each instance, ligation of RAGE by either AGEs or amyloid- β peptide results in enhanced cellular oxidant stress, based on multiple criteria, including oxidant-sensitive nuclear translocation of the transcription factor NF- κ B. The current study has indicated that, in

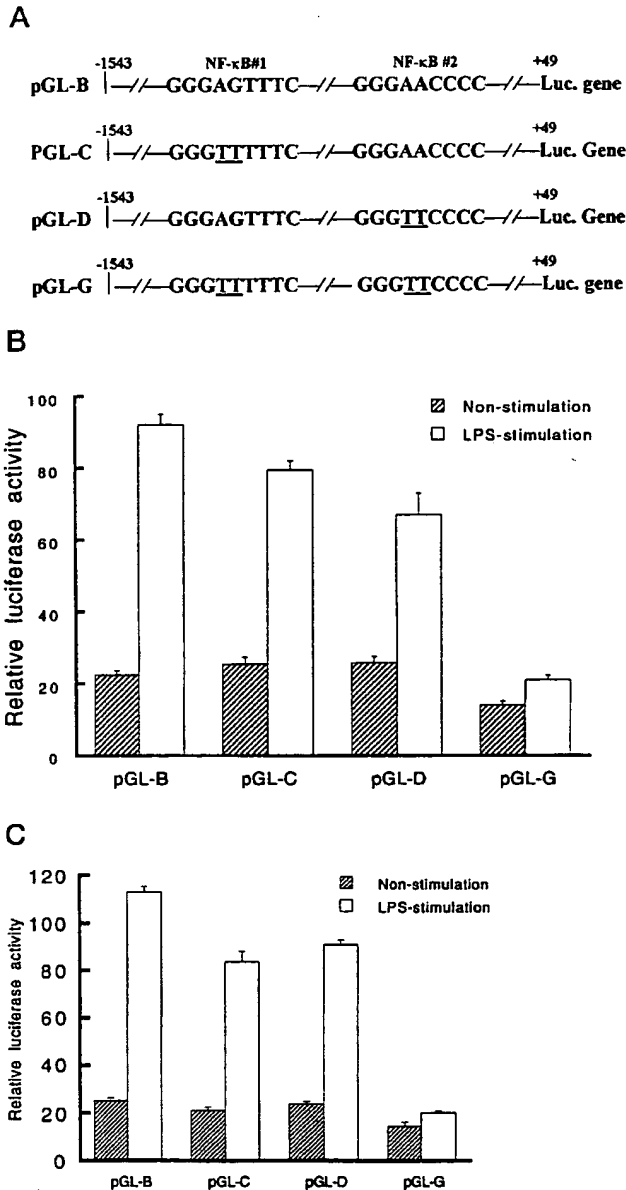


FIG. 9. PCR-directed mutagenesis (A) and relative luciferase activities of promoter luciferase chimeras expressed in BAECs (B) or RSMCs (C). A, PCR-directed mutagenesis was performed as indicated in the figure for NF- κ B binding sites 1 and 2 in the RAGE promoter by substitution of the nucleotides (*underlined*) in each of the sites. The original sequences for the two NF- κ B binding sites identified in the RAGE promoter are shown in the wild-type construct (pGL-B), and the mutated sequences are shown in constructs pGL-C, pGL-D and pGL-G. B and C, relative luciferase activities from BAECs (B) or RSMCs (C) under basal conditions (cross-hatched bar) or in the setting of stimulation with LPS (open bar) after normalization for galactosidase activity. Data represent the mean \pm standard error of at least three separate experiments. In B (BAECs), basal conditions were as follows: pGL-B versus pGL-C, $p > 0.05$; pGL-B versus pGL-D, $p > 0.05$; pGL-B versus pGL-G, $p < 0.01$. Stimulatory conditions (LPS) were: pGL-B versus pGL-C, $p < 0.05$; pGL-B versus pGL-D, $p < 0.05$; pGL-B versus pGL-G, $p < 0.01$. All differences between stimulation with LPS and basal conditions are significant (in pGL-B, -C, and -D, $p < 0.01$; pGL-G (LPS) versus pGL-G (basal), $p < 0.05$). In C (RSMCs), basal conditions were as follows: pGL-B versus pGL-C, $p > 0.05$; pGL-B versus pGL-D, $p > 0.05$; pGL-B versus pGL-G, $p < 0.05$. Stimulatory conditions (LPS) were: pGL-B versus pGL-C, $p < 0.05$; pGL-B versus pGL-D, $p < 0.05$; pGL-B versus pGL-G, $p < 0.01$. All differences between stimulation with LPS and basal conditions are significant (in pGL-B, -C, and -D, $p < 0.01$; pGL-G (LPS) versus pGL-G (basal), $p < 0.05$).

addition, expression of RAGE itself can be regulated by NF- κ B. This is especially relevant to the high expression of RAGE observed in certain diabetic tissues, Alzheimer's brain, and in inflammatory lesions as well. Although future studies will be required to extrapolate our findings concerning RAGE regulation to the *in vivo* setting, we speculate that the two functional NF- κ B sites within the RAGE promoter are critical for regulation of RAGE expression in certain pathologic states.

Our previous studies demonstrated that RAGE is expressed to varying degrees at basal/enhanced levels in endothelial cells, vascular smooth muscle cells, mononuclear phagocytes, mesangial cells, and certain neurons (15), depending on the existence of such conditions as hyperglycemia, oxidant stress, neuronal development, or inflammation as considered above. However, for example, RAGE is not expressed to any significant degree in most epithelial cells. These data suggested that negative regulatory elements exist within this promoter, which are responsible for suppressing its expression in certain cell types, as well as maintaining low levels of expression in endothelial cells and vascular smooth muscle cells under basal conditions. In these initial studies, we have identified the region -1543/-587 as important in mediating promoter responsiveness to the prototypic stimulus, LPS. In contrast, constructs encompassing the -587/+49 region do not appear responsive to any significant degree to the stimulus tested in the present work. The -202/+49 construct, furthermore, had a very low promoting function, suggesting either that negative regulatory elements may be located within this region which impact upon the ability of the promoter to respond to such stimuli, or that NF- κ B-like binding sites are essential for the ability of this promoter to respond to the prototypic stimulus LPS. Further studies will be required to dissect the role of these and other putative negative regulatory elements within this promoter as this relates to variable basal expression and inducibility in diverse cell types.

An interesting finding elucidated in these studies concerns the observation that the RAGE promoter likely belongs to the family of TATA-less promoters (40). Sequence analysis of the RAGE promoter revealed no typical CAAT and TATA boxes at appropriate positions upstream of the transcriptional start site (usually within 25 bp of this site; Ref. 40). Other genes whose expression is regulated in a tissue-specific manner and that lack TATA consensus sequences have been identified, such as synapsin I (41), brain-specific aldolase C (42), nerve growth factor (43), leukocyte integrin α subunit (44), and lymphocyte CD4 (45). Despite the finding that TATA-less promoters are often associated with so-called "housekeeping genes," other such genes have been demonstrated to be controlled by complex regulatory mechanisms (40). Indeed, the present data and previous observations concerning RAGE expression support its addition to the latter subset of TATA-less promoters.

Taken together, these data support the concept that the transcriptional/translational regulation of RAGE expression is strictly controlled under homeostatic conditions as well as in stimulatory conditions such as diabetes, inflammatory arthropathies associated with dialysis, neuronal oxidant stress, and neurite outgrowth in the developing central nervous system. Toward this end, studies are under way to dissect the role of other putative transcription factor binding sites as well as silencing/enhancing regulatory elements in diverse cell types, in both basal and pathologic states.

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